PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : **C07K 7/10, A61K 37/43**	**A1**	**(11) International Publication Number:** **WO 90/15821** **(43) International Publication Date:** 27 December 1990 (27.12.90)

(21) International Application Number: PCT/US90/02923

(22) International Filing Date: 30 May 1990 (30.05.90)

(30) Priority data:

368,231	16 June 1989 (16.06.89)	US
427,868	27 October 1989 (27.10.89)	US

(60) Parent Application or Grant
(63) Related by Continuation
US 427,868 (CIP)
Filed on 27 October 1989 (27.10.89)

(71) Applicant *(for all designated States except US):* THE UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KUBIAK, Teresa, M. [PL/US]; 1523 Kickapoo Court, Kalamazoo, MI 49007 (US). FRIEDMAN, Alan, R. [US/US]; 7646 Wendy Lane, Portage, MI 49002 (US).

(74) Agent: JAMESON, William, G.; The Upjohn Company, Kalàmazoo, MI 49001 (US).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: STABILIZED, POTENT GRF ANALOGS

(57) Abstract

Novel GRF peptides with enhanced stability in plasma and aqueous environments at neutral pH.

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MC**	Monaco
AU	Australia	**FI**	Finland	**MG**	Madagascar
BB	Barbados	**FR**	France	**ML**	Mali
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Fasso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GR**	Greece	**NL**	Netherlands
BJ	Benin	**HU**	Hungary	**NO**	Norway
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**SD**	Sudan
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CG	Congo			**SN**	Senegal
CH	Switzerland	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
DE	Germany, Federal Republic of	**LK**	Sri Lanka	**TG**	Togo
DK	Denmark	**LU**	Luxembourg	**US**	United States of America

STABILIZED, POTENT GRF ANALOGS

INTRODUCTION

The present invention relates to a peptide having influence on the function of the pituitary gland in humans and other animals, particularly mammals. In particular, the present invention is directed to peptides which promote the release of growth hormone by the pituitary gland. The peptides of the present invention are potent in vivo, more stable in plasma and selected peptides are more stable in an aqueous environment at neutral pH than native GRF sequences.

BACKGROUND OF THE INVENTION

Physiologists have long recognized that the hypothalamus controls the secretory functions of the adenohypophysis with the hypothalamus producing special substances which stimulate or inhibit the secretion of each pituitary hormone. In 1982, human pancreatic (tumor) releasing factors (hpGRF) were isolated from extracts of human pancreatic tumors, purified, characterized, synthesized, tested, and found to promote release of growth hormone (GH) by the pituitary. Guillemin, R., et al., Science 218, 585-585 (1982). Since then, corresponding hypothalamic GH releasing factors from other species including the rat species, the porcine species, the ovine species, the bovine and caprine species and from the human species have also been characterized and synthesized.

Human hypothalamic GRF (hGRF) has been found to have the same formula as hpGRF, namely: H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2.

Rat GRF (rGRF) has been found to have a Ser residue at position 8 and the formula: H-His-Ala-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Arg-Ile-Leu-Gly-Gln-Leu-Tyr-Ala-Arg-Lys-Leu-Leu-His-Glu-Ile-Met-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Arg-Ser-Arg-Phe-Asn-OH. (See for example US Patent 4,595,676).

Bovine GRF (bGRF) has been found to have the formula: H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Asn-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu-NH_2.

Porcine GRF has been found to have a Ser residue at position 28.

It has been reported that native GRF sequences are subject to rapid inactivation by blood plasma enzymes. The rapid breakdown involves cleavage of the 2-3 bond of the peptide by a dipeptidyl-peptidase, Type IV (DPP-IV), which in the past was also named dipeptidylaminopeptidase-IV. Frohman, L.A. et al., J. Clin. Invest., 78, 906-913 (1986).

The metabolic stability of GRF and various methods for protecting GRF peptides against dipeptidylpeptidase cleavage have been proposed, including Felix et al., Synthesis and biological activity of novel linear and cyclic GRF analogs, in Peptides. Chemistry and Biology, Proc. 10th Am. Peptide Symposium, Ed. G.R. Marshall, ESCOM Sci. Publishers, Leiden, The Netherland, pp.465-467, (1988), who reported on GRF analogs substituted with desNH2-Tyr at position 1, or/and D-Ala at position 2 which had enhanced stability of their N-termini to enzymatic degradation. This information was recently confirmed by Frohman et al., Dipeptidylpeptidase-IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma. J. Clin. Invest. 83, 1533-1540 (1989). In addition, the latter group showed that N-acetylation and N-methylation of the N-terminal tyrosine residue or substitution with D-Tyr-1 in GRF completely inhibited cleavage at the 2-3 position. On the other hand, alpha-methylation of Tyr-1, only partially blocked degradation by DPP-IV.

Murphy, W.A. and Coy, D.H., Potent long-acting alkylated analogs of growth hormone-releasing factor, Peptide Research 1, 36-41 (1988), describe analogs of GRF which show enhanced resistance to enzymatic degradation as a result of N-alkylation or N-arylalkylation of the N-terminal amino acid with or without concomitant N-alkylation of the side groups of lysines within the peptide chain.

Native GRF sequences have a Gly residue at the 15-position. Analogs with Ala or Leu at the 15-position are known to have increased GH releasing potency. See for example US Patents 4,649,131 and 4,734,399 as well as Ling, N., et al., Quo Vadis?, Symposium, Sanofi Group, May 29-30, 1985, Toulouse-Labege, France (pp. 309-322). Substitutions of Gly-15 with Val or alpha-amino-isobutiric acid also resulted in enhanced potency of GRF analogs, Felix et al., Synthesis and biological activity of novel growth hormone releasing factor analogs, in Peptides 1986, Walter de Gruyter & Co., Berlin-New York,

pp. 481-484 (1987).

A.M. Felix has reported on a program to synthesize analogs with enhanced and/or prolonged biological activity, including the preparation and testing of Ala^{15} h-GRF(1-29)NH_2 and desNH$_2$-Tyr^1, D-Ala^2, Ala^{15} hGRF(1-29)NH_2. See, for example, U.S. Patents 4,649,131 and 4,734,399 as well as A.M. Felix, E.P. Heimer, T.F. Mowles, H. Bisenbeis, P. Leung, T.J. Lambros, M. Ahmad, C.-T. Wang & Paul Brazeau: Synthesis and biological activity of novel growth hormone releasing factor analogs. in Peptides 1986, pp. 481-484 (1987); Felix, A.M., Wang, C.T., Heimer, E., Fournier, A., Bolin, D., Ahmad, M., Lambros, T., Mowles, T., and Miller, L.: Synthesis and biological activity of novel linear and cyclic GRF analogs. in Peptides. Chemistry and Biology. Proceedings of the 10th American Peptide Symposium, Ed. G.R. Marshall, Escom Science Publishers, Leiden, The Netherlands (1988), pp. 465-467; D.Peticlerc, H. Lapierre, G. Pelletier, P. Dubreuil, P. Gaudreau, T. Mowles, A. Felix and P. Brazeau: Effect of a potent analog of human growth hormone-releasing factor (hGRF) on growth hormone (GH) release and milk production of dairy cows. Meeting Abstract P223, 82nd Meeting American Dairy Sci. Assn., Columbia, MO, June 21-24 (1987).

A GRF analog that was modified with Ser^2, in addition to eight other modifications in the same molecule, is described by Tou et al., Amphiphilic Growth Hormone-Releasing Factor (GRF) analogs: peptide design and biological activity in vivo. Biochem. Biophys. Res. Commun. 139, 763-770 (1986). This analog was reported to have 165% activity in vivo in sheep as compared to bGRF(1-44)NH2.

U.S. Patent 4,734,399 discloses GRF analogs having Ala, N-Methyl-D-Ala or D-Ala at position 2 and Ala, Leu, Val, Ile, Nle, Nval, β-Ala or α-Ala at position 15. See also U.S. Patent 4,649,131.

European Patent Application of Coy and Murphy, Publication Number 0 188 214, Application Number 86100127.9, discloses GRF analogs with Leu or Phe at position 2, in addition to GRF peptides having various unnatural amino acids of L or D-configuration as substituents at position 2.

GRF analogs with very low bioactivity having Sar^2 or Pro^2 are described by Coy et al., Strategies in the design of synthetic agonists and antagonists of growth hormone-releasing factor, Peptides, vol. 7, Suppl. 49-52 (1986).

Ling et al., Growth hormone-releasing factor analogs with potent antagonistic activity. in Peptides. Chemistry and Biology. Proceedings of the 10th American Peptide Symposium, Ed. G.R. Marshall, Escom Science Publishers, Leiden, The Netherlands (1988), pp. 484-486, reported on a series of GRF analogs substituted with either Arg or a variety of D-amino acids at position 2. All of them are less potent than the parent hormone and some of them displayed antagonistic activity.

The prior invention of a co-worker provides synthetic GRF polypeptides having a Ser residue in place of the amino acid residue normally found at position 8 and 28 of the polypeptide as a means of inhibiting chemical breakdown (deamidation) in aqueous environments. See U.S. Patent Application Serial No. 07/303,518, filed 27 January 1989 and Serial No. 07/323,955, filed 15 March 1989.

The prior invention of a co-worker provides synthetic GRF polypeptides having a cysteic acid residue (Cya) substituted for the amino acid residue in position R_3 and/or R_{25}. See U.S. Patent Application Serial No. 07/150,301, filed 29 January 1988 and Serial No. 89/00245, filed 27 January 1989.

A 29-amino acid analog of hGRF was designed by G. Velicelebi, et al., Proc. Natl. Aca. Sci USA, Vol 83, 5397-5399 (1986), in which the sequence of the first six amino acids at the amino terminus, and differing from the natural peptide by 13 amino acid in the rest of the sequence including incorporation of a Ser residue at position 8. The amide and free acid forms of the analog had the formula: H-Tyr-Ala-Asp-Ala-Ile-Phe-Ser-Ser-Ala-Tyr-Arg-Arg-Leu-Leu-Ala-Gln-Leu-Ala-Ser-Arg-Arg-Leu-Leu-Gln-Glu-Leu-Leu-Ala-Arg-NH_2/OH. When assayed for the ability to stimulate growth hormone (GH) secretion in primary cultures of rat anterior pituitary cells, the amide analog was 1.57 times as potent as hGRF(1-40)OH, while the free acid form was reported to be 1/6th as potent in the same assay.

Vale, et al., (US Patent Application Serial No. 053,233, filed May 22, 1987) describe 31-residue hGRF analogues which utilize a 31-position residue possessing a functional side chain group which may be conjugated to a separate protein. The 31-residue hGRF analogues may also have substitutions for other residues which appear in a natural GRF sequence, such as Asn or Ser in the 8-position, Phe in the 10-position, or Ala in the 15-position. Asn or Ser may be

present in the 28-position.

Asn residues in polypeptides are reported to be the subject, under some circumstances, to deamidation in the presence of water. However, the rules governing the rates of deamidation are not clear. For example, in the polypeptide trypsin only some of the Asn residues, with the partial sequence Asn-Ser, are deamidated while others are not. See Kossiakoff, AA, Science 240, 191-194 (1988).

European Patent Application Number 86308337.4, Publication number 0 220 958, discloses a class of compounds having the formula H-X-Pro-Peptide in which X is the residue of a naturally occurring amino acid, Pro refers to the naturally occurring amino acid proline and Peptide is a sequence of amino acid residues defining that of a biologically active peptide or protein. Examples of H-X-Pro-Peptide include Met-Pro-(growth hormone-releasing factor) which can be chemically converted to GRF.

N-terminally extended analogs on non-GRF peptides have been reported for various purposes including, for example:

M.A. Tallon et al., Biochem., 26:7767-7774 (1987), made synthetically a series of N-terminally extended analogs of yeast alpha-mating factor with Ala, Glu-Ala, Ala-Glu-Ala or Glu-Ala-Glu-Ala in the extension part. These peptides were used in structure-activity relationship studies.

D. Andreu et al., 20th Eur. Peptide Symp., Tubingen, GFR, September 4-9, 1988, Symposium Abstracts, p. 33, synthesized the entire 64-amino acid sequence of the precursor form of cecropin A along with several shorter peptides corresponding to potential processing intermediates. Among them there was a full cecropin sequence extended with Ala-Pro-Gly-Pro at its N-terminus which was used to show that the extension part was indeed cleaved by a partially purified dipeptidylpeptidase-like enzymatic preparation obtained from the cecropia silkmoth pupa. See also H. Boman et al. J. Biol. Chem. 264:5852-5860 (1989).

G. Kreil et al., Eur. J. Biochem., 111:49-58 (1980) reports that melittin, the main constituent of honeybee venom, is derived from pro-melittin. The pro-sequence of pro-melittin consists of six X-Pro and five X-Ala repetitive dipeptidyl residues. The results presented by Kreil et al. suggest that the precursor-product conversion may proceed via stepwise cleavage of dipeptide units by a dipeptidylpep-

tidase IV type enzyme present in extracts from venom glands.

C. Mollay et al., Eur. J. Biochem., 160:31-35 (1986). Caerulein and xenopsin are two peptides found in skin secretion of Xenopus laevis. The former has a sequence of Phe-Ala-Asp-Gly and the latter Ser-Ala-Glu-Ala in the N-terminal extensions in their respective precursor forms. A dipeptidylpeptidase of type IV, isolated from frog skin secretion, has the specificity required for the cleavage of these N-terminal extensions leading to the formation of the mature products.

D. Julius et al., Cell, 32:839-852 (1983). Alpha factor mating pheromone is a peptide of 13 amino acids secreted by Saccharomyces cervisiae alpha cells. Nonmating alpha-cell mutants, which lack a membrane-bound dipeptidylpeptidase, do not produce normal alpha-factor, but release a collection of incompletely processed forms with structures Glu-Ala-Glu-Ala-alpha-factor or Asp-Ala-Glu-Ala-alpha-factor that have a markedly reduced biological activity. It has been shown that the membrane-bound dipeptidylpeptidase is required for normal alpha-factor precursor processing and this process may be rate-limiting for alpha-factor maturation in normal yeast alpha cells.

C.L. Choy et al., Eur. J. Biochem., 160:267-272 (1986). The prosequence of the antifreeze protein from the Newfoundland winter flounder contains four X-Pro and seven X-Ala repetitive sequences in its N-terminal part. Although the processing of this precursor has not been investigated, the authors speculate that such a conversion might take place in serum by a dipeptidylpeptidase-line enzyme which would sequentially cleave the dipeptidyl units in the extension part to release the mature antifreeze protein.

Subsequent to the priority date of the parent application, Suhr et. al. reported the isolation and characterization of a full-length cDNA clone encoding mouse GRF. The mature mouse GRF was predicted to be a 42 amino acid residue peptide with a free carboxyl-terminus. This peptide has a valine residue at position 2 which makes it unique among the GRFs from other species all having Ala at position 2. See Mol. Endocrinology 3: 1693-1700, 1989.

SUMMARY OF THE INVENTION

The present invention provides a synthetic polypeptide which promotes the release of growth hormone by the pituitary gland (GRF

PEPTIDE) and having Thr, Val or Ile residue in place of the amino acid residue normally found at position 2 in combination with one of the following amino acids Ala, Val, Leu, Ile or Gly at position 15. Optionally, the GRF PEPTIDE can have a Ser residue in place of the amino acid residue normally found at position 8 and 28 of the polypeptide. In addition, the GRF PEPTIDES of the present invention can optionally be N-terminally extended with C_1-C_5 alkyl, benzyl, H-$(Y\text{-}X)_n$ or H-$(Y\text{-}X)_m(Y'\text{-}X')_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid, preferably Tyr or Asp; X and X', being the same or different, is selected from Thr, Ser or Ala, preferably Thr or Ser; n is 1-10; m is 1-5; p is 1-5.

The peptides of the present invention are potent in vivo and more stable than native GRF sequences against breakdown by blood plasma enzymes. In addition, compounds substituted with Ser^8 and Ser^{28} are protected against deamidation in aqueous environments and are chemically more stable.

DETAILED DESCRIPTION OF THE INVENTION

The term "GRF PEPTIDE", as used in the specification and claims, means a known polypeptide which is between about 20 and 44 residues, preferably between 27 and 44 residues, in length and that promotes the release of growth hormone by the pituitary gland. Illustrative GRF PEPTIDES include the natural or synthetic polypeptides disclosed in US Patent Nos. 4,517,181, 4,518,586, 4,528,190, 4,529,595, 4,563,352, 4,585,756, 4,595,676, 4,605,643, 4,610,976, 4,626,523, 4,628,043, 4,689,318, and U.S. Patent Application Serial No. 89/00245, filed 27 January 1989; all of which are incorporated herein by reference. Felix, A., Wang, C.T., Heimer, E., Fournier, A., Bolin, D., Ahmed, M., Lambros, T., Mowles, T., and Miller, L., "Synthesis and Biological Activity of Novel Linear & Cyclic GRF Analogs", in Peptides. Chemistry and Biology, Proc. 10th Am. Peptide Symposium, Ed. G.R. Marshall, ESCOM Sci. Publishers, Leiden, The Netherland, pp.465-467, (1988); Tou, J.S., Kaempfe, L.A., Vineyard, B.D., Buonomo, F.C., Della-Fera, M.A., and Baile, C.A., "Amphiphilic Growth Hormone Releasing Factor Analogs. Peptide Design and Biological Activity in vivo", Biochem. Biophys. Res. Commun. 139 #2, pp. 763-770 (1986); Coy, D.H., Murphy, W.A., Sueires-Diaz, J., Coy, E.J., Lance, V.A., "Structure Activity Studies on the N-Terminal Region of Growth Hormone Releasing Factor", J. Med. Chem. 28, pp.

181-185 (1985); Felix, A.M., Heimer, E.P., Mowles, T.F., Eisenbeis, H., Leung, P., Lambros, T.J., Ahmed, M., and Wang, C.T., "Synthesis and Biological Activity of Novel Growth Hormone Releasing Factor Analogs", in Peptides 1986, Walter de Gruyter & Co., Berlin-New York, pp. 481-484 (1987); Velicelebi, G., Patthi, S., and Kaiser, E.T., "Design and Biological Activity of Analogs of Growth Hormone Releasing Factor with Potential Amphiphilic Helical Carboxyl Termini", Proc. Natl. Acad. Sci. U.S.A., 85, pp. 5397-5399 (1986); Ling., N., Baird, A., Wehrenberg, W.B., Munegumi, T., and Ueno, N., "Synthesis GRF Analogs as Competitive Antagonists of GRF Therapeutic Agents Produced by Genetic Engineering", Quo Vadis Symposium, Sanofi Group, May 29-30, 1985, Toulouse-Labege, France, pp. 309-329. Murphy, W.A. and Coy, D.H., Potent long-acting alkylated analogs of growth hormone-releasing factor, Peptide Research 1, 36-41 (1988). J.C Tou, L.A. Kaempfe, B.D. Vineyard, F.C. Buonomo, M.A. Della-Fera and C.A. Baile: Amphiphilic Growth Hormone-Releasing Factor (GRF) analogs: peptide design and biological activity in vivo. Biochem. Biophys. Res. Commun. 139, 763-770 (1986). The term GRF PEPTIDE includes nontoxic salts thereof.

The nomenclature used to define the GRF PEPTIDE is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965) wherein in accordance with conventional representation the amino group at the N-terminal appears to the left and the carboxyl group at the C-terminal to the right. Where the amino acid residue has isomeric forms, the L-form of the amino acid is being represented unless otherwise expressly indicated.

The present invention provides synthetic GRF peptide analogs (GRF PEPTIDES) having the following formula:

R-R_1-R_2-R_3-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-R_{12}-R_{13}-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-R_{21}-R_{22}-Leu-Gln-R_{25}-Ile-R_{27}-R_{28}-Arg-Gln-Gln-Gly-Glu-R_{34}-R_{35}-Gln-Glu-R_{38}-R_{39}-R_{40}-Arg-R_{42}-Arg-Leu-Z

wherein

R is H, C_1-C_5 alkyl, benzyl, H-(Y-X)$_n$ or H-(Y-X)$_m$(Y'-X')$_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid, preferably Tyr or Asp; X and X', being the same or different, is selected from Thr, Ser or Ala, preferably Thr or Ser; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His, preferably Tyr;

R_2 is Thr, Val or Ile, preferably Val or Ile;

R_3 is Asp or Cya, preferably Asp;

R_8 is Asn or Ser, preferably Ser;

R_{12} is Lys, N-ε-alkyl- or N-ε-benzyl-Lys or Arg, preferably Lys; or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{13} is Val or Ile, preferably Val;

R_{15} is Ala, Val, Leu, Ile or Gly (preferably Ala, Val, Leu or Ile, more preferably Ala);

R_{18} is Ser or Tyr, preferably Ser;

R_{21} is Lys, N-ε-alkyl- or N-ε-benzyl-Lys or Arg, preferably Lys or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{22} is Ala or Leu, preferably Leu;

R_{25} is Asp or Glu, preferably Asp;

R_{27} is Met, Ile or Leu, preferably Leu or Ile;

R_{28} is Asn or Ser, preferably Ser;

R_{34} is Ser or Arg, preferably Arg;

R_{35} is Asn or Ser, preferably Asn;

R_{38} is Arg or Gln, preferably Gln;

R_{39} is Gly or Arg, preferably Gly;

R_{40} is Ala or Ser, preferably Ala;

R_{42} is Ala, Val or Phe, preferably Val; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical $-COOR_a$, $-CR_aO$, $-CONHNHR_a$, $-CON(R_a)(R_b)$ or $-CH_2OR_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 (preferably 27 through 44) as its C-terminus; or a Hse(lactone), HseOH or $HseN(R_a)(R_b)$ of the foregoing and/or a non-toxic salt of the foregoing.

R is preferably hydrogen (H).

Examples of C_1-C_8 alkyl are methyl, ethyl, propyl, butyl, pentyl, hexyl, octyl and isomeric forms thereof.

The term iPr refers to isopropyl.

The term Bzl refers to benzyl.

An embodiment of this invention is the peptide Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2 having the formula: H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH_2.

A further embodiment of this invention is the peptide Val^2 Ala^{15}

Leu^{27} bGRF(1-29)NH_2 having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH_2.

Still another embodiment of this invention is the peptide Ile^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2 having the formula: H-Tyr-Ile-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH_2.

A further embodiment of this invention is the peptide Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-44)NH_2 having the formula: H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu-NH_2.

Preferred embodiments of this invention are the peptides Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Val^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, or a non-toxic salt thereof.

More preferred embodiments of this invention are the peptides Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, or a non-toxic salt thereof.

Another embodiment of this invention is the peptide N-α-iPr-Tyr^1 Thr^2 N-ε-iPr-$Lys^{12,21}$ Ala^{15} Leu^{27} Hse^{30} bGRF(1-30)NH(C_2H_5) having the formula: N-α-iPr-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-

Arg-N-ε-iPr-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-N-ε-iPr-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-HseNH(Ethyl).

Still another embodiment of this invention are N-terminally extended peptides, including for example N-α-(Tyr-Ser)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Tyr-Ser-Tyr-Thr)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-$(Tyr\text{-}Thr)_2$-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse ^{32} bGRF(1-32)NH_2; N-α-(Tyr-Ser)-Tyr^1 Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Tyr-Ser-Tyr-Thr)-Tyr^1 Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Asp-Ala-Tyr-Thr)-Tyr^1 Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Asp-Ala)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-37)NH_2; N-α-$(Tyr\text{-}Thr)_2$-Tyr^1 Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse ^{32} bGRF(1-32)NH_2; N-α-(Tyr-Ser)-Tyr^1 Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Tyr-Ser-Tyr-Thr)-Tyr^1 Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-$(Tyr\text{-}Thr)_2$-Tyr^1 Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2; N-α-(Asp-Ala)-Tyr^1 Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-37)NH_2.

Another embodiment of this invention is any of the foregoing embodiments wherein Cya is substituted for Asp in position 3 and/or 25, preferably in position 3.

Evidence of the improved metabolic stability of the compounds of this invention (when compared to native GRF) is illustrated in Table I by the in vitro stability data provided. It is noteworthy that in bovine plasma in vitro, the analogs having Thr^2, Val^2, Ile^2 or Leu^2 were not cleaved after residue 2 by plasma DPP-IV under the experimental conditions used in the study (one hour plasma incubation). However, on extended plasma incubation, Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NNH_2 exhibited slight DPP-IV related cleavage.

In addition, the enhanced in vivo potency of the compounds of this invention is illustrated in Table I. For example, a person skilled in the art would recognize from the relative in vivo potency (at a dose of 0.01 nmol/kg) that Ile2 Ala15 Leu27 bGRF(1-29)NH2, trifluoroacetate salt would be expected to exhibit a potency of 10-20 times that of its isomer Leu2 Ala15 Leu27 bGRF(1-29)NH2, trifluoroacetate salt when the ED50 of each is determined and compared.

For purposes of commercial production methodology, the carboxy terminal residue is preferably homoserine, homoserine lactone, homoserine amide, or a C_1-C_8 alkyl (preferably C_1-C_4 alkyl),

secondary or tertiary amides of homoserine.

The synthetic GRF peptide analogs are synthesized by a suitable method, including for example the methods disclosed in U.S. Patent 4,529,595 (Col 2, ln 35 to Col 5, ln 64) and US Patent 4,689,318 (Col 2, ln 23 to Col 9, ln 13), each of which are incorporated herein by reference.

Procedure A sets forth a method for synthesizing GRF peptide analogs of the subject invention.

PROCEDURE A

The peptides are synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, California) and synthesis cycles supplied by Applied Biosystems. Boc Amino acids and other reagents were supplied by Applied Biosystems and other commercial sources. Sequential Boc chemistry using double couple protocols are applied to the starting p-methyl benzhydryl amine resin for the production of C terminal carboxamides. For the production of C terminal acids, the corresponding PAM resin is used. Asparagine, glutamine, and arginine are coupled using preformed hydroxy benztriazole esters. All other amino acids are coupled using the preformed symmetrical Boc amino acid anhydrides.

The following side chain protection is used:

Arg, Tosyl
Asp, Benzyl
Cys, 4-Methyl Benzyl
Glu, Benzyl
Ser, Benzyl
Thr, Benzyl
Tyr, 4-Bromo Carbobenzoxy
Lys, 2-Chloro Carbobenzoxy

Boc deprotection is accomplished with trifluoroacetic acid (TFA) in methylene chloride. When Hse containing analogs are desired, Met should be incorporated by solid phase and then modified with cyanogen bromide after HF cleavage by methods well known in the art. This cyanogen bromide cleavage converts the Met to the C-terminal Hse lactone peptide. This can be converted to the Hse amide peptide by treatment with the appropriate amine in a solvent such as methanol or dimethyl formamide. Following completion of the

synthesis, the peptides are deprotected and cleaved from the resin with anhydrous hydrogen fluoride containing 10% p-cresol. Cleavage of the side chain protecting group(s) and of the peptide from the resin is carried out at 0°C or below, preferably -20°C for thirty minutes followed by thirty minutes at 0°C. After removal of the HF, the peptide/resin is washed with ether, and the peptide extracted with glacial acetic acid and lyophilized. Before purification, crude cysteine containing peptides are then oxidized to the corresponding cysteic acid containing compound using performic acid at -10°C to 10°C, preferably at 0°C, as described by Stewart et al., Solid Phase Peptide Synthesis, pg. 113, Pierce Chemical Company, Rockford, Illinois, 1984. Conversion to C-terminal Hse lactones and Hse amides is carried out as described above.

Purification is carried out by ion exchange chromatography on a Synchroprep S-300 (SynChrom Inc. Linden, Indiana) cation exchange column. The peptide is applied using a buffer of 20 millimolar TRIS (pH 6.8) in 20% acetonitrile and eluted using a gradient of 0-0.3 molar sodium chloride in the same solvent. Compounds are further purified and desalted by reverse phase liquid chromatography on a Vydac C-18 (Separations Group, Hesperia, California) column using water:acetonitrile gradients, each phase containing 0.1% TFA. The desired fractions are pooled and lyophilized yielding the desired GRF PEPTIDE as its trifluoroacetate salt. The trifluoroacetate salt can be converted, if desired to other suitable salts, by well known ion exchange methods.

Peptides are hydrolyzed under vacuum by a vapor phase method in a Pico-Tag Work Station (Waters) using constant boiling HCl (Pierce) in the presence of phenol as scavenger at 110 C for 24 hrs. Hydrolysates are analyzed on a Beckman Amino Acid Analyzer, Model 6300. Peptide content is calculated using norleucine at a known concentration as an internal standard.

EXAMPLES

Example 1: Preparation of Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 1

The synthesis of the GRF analog peptide having the formula: H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A.

Amino acid analysis, theoretical values in parentheses: Asp 3.96 (4); Thr 1.84 (2); Ser 1.79 (2); Glu 2.01 (2), Ala 3.04 (3); Val 0.99 (1), Ile 1.93 (2), Leu 5.05 (5); Tyr 1.96 (2); Phe 0.96 (1); Lys 2.02 (2); Arg 3.09 (3).

Example 2: Preparation of Val^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 2

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.11 (4); Thr 0.96 (1); Ser 1.79 (2); Glu 2.11 (2), Ala 3.04 (3); Val 2.1 (2), Ile 1.91 (2), Leu 5.17 (5); Tyr 2.00 (2); Phe 0.98 (1); Lys 1.99 (2); Arg 2.70 (3).

Example 3: Preparation of Ile^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 3.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Ile-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.12 (4); Thr 0.97 (1); Ser 1.75 (2); Glu 2.09 (2), Ala 3.00 (3); Val 1.05 (1), Ile 2.91 (3), Leu 5.16 (5); Tyr 2.00 (2); Phe 0.99 (1); Lys 1.98 (2); Arg 2.75 (3).

Example 4: Preparation of N-α-(Tyr-Thr)Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 4.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Thr-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.05 (4); Thr 2.68 (3); Ser 1.77 (2); Glu 2.07 (2), Ala 2.90 (3); Val 1.08 (1), Ile 1.89 (2), Leu 5.20 (5); Tyr 2.87 (3); Phe 0.93 (1); Lys 2.01 (2); Arg 3.07 (3).

Example 5: Preparation of N-α-$(Tyr\text{-}Thr)_2Tyr^1$ Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 5.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Thr-Tyr-Thr-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-

Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.06 (4); Thr 3.66 (4); Ser 1.85 (2); Glu 2.05 (2), Ala 2.93 (3); Val 1.09 (1), Ile 1.91 (2), Leu 5.15 (5); Tyr 3.91 (4); Phe 0.95 (1); Lys 2.00 (2); Arg 3.04 (3).

Example 6: Preparation of N-α-(Tyr-Thr)Tyr^{1} Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 6.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Thr-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.06 (4); Thr 1.86 (2); Ser 1.77 (2); Glu 2.07 (2), Ala 3.98 (4); Val 1.08 (1), Ile 1.89 (2), Leu 5.14 (5); Tyr 2.94 (3); Phe 0.96 (1); Lys 1.99 (2); Arg 3.04 (3).

Example 7: Preparation of N-α-(Tyr-Thr)Tyr^{1} Ile^{2} Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 7.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Thr-Tyr-Ile-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.07 (4); Thr 1.87 (2); Ser 1.75 (2); Glu 2.07 (2), Ala 2.94 (3); Val 1.09 (1), Ile 2.87 (3), Leu 5.12 (5); Tyr 2.92 (3); Phe 0.96 (1); Lys 2.00 (2); Arg 3.05 (3).

Example 8: Preparation of N-α-(Tyr-Ser)Tyr^{1} Thr^{2} Ala^{15} Leu^{27} bGRF(1-29)NH_2, trifluoroacetate salt; Cpd. No. 8.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Ser-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Asn-Arg-NH2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 4.11 (4); Thr 1.82 (2); Ser 2.64 (3); Glu 2.05 (2), Ala 2.90 (3); Val 1.04 (1), Ile 1.87 (2), Leu 5.16 (5); Tyr 2.92 (3); Phe 0.94 (1); Lys 2.01 (2); Arg 3.04 (3).

Example 9: Preparation of Val^{2} Ser^{8} Ala^{15} Leu^{27} Ser^{28} Hse^{45} bGRF(1-45)NH2, trifluoroacetate salt Compound No. 9.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Glu-Arg-Asn-Gln-Glu-Gln-Gly-Ala-Lys-Val-Arg-Leu-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 3.05 (3); Thr 0.93 (1); Ser 3.20 (4); Glu 8.41 (8); Gly 2.04 (2); Ala 4.17 (4); Val 2.87 (3); Ile 1.79 (2); Leu 5.96 (6); Tyr 1.88 (2); Phe 0.83 (1); Lys 2.94 (3); Arg 4.93 (5). Mass spectrum $(M+H)^+$, found 5179.3, theoretical 5179.9

Example 10: Preparation of Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, trifluoroacetate salt Compound No. 10.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 2.04 (2); Thr 0.93 (1); Ser 3.23 (4); Glu 4.19 (4); Gly 0.95 (1); Ala 3.21 (3); Val 1.93 (2); Ile 1.82 (2); Leu 4.96 (5); Tyr 1.91 (2); Phe 0.91 (1); Lys 1.99 (2); Arg 3.03 (3). Mass spectrum $(M+H)^+$, found 3770.1, theoretical 3770.4

Example 11: Preparation of Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, trifluoroacetate salt Compound No. 11.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 2.05 (2); Thr 0.96 (1); Ser 3.32 (4); Glu 2.04 (2); Ala 3.10 (3); Val 2.12 (2); Ile 1.86 (2); Leu 5.04 (5); Tyr 1.91 (2); Phe 0.89 (1); Lys 2.06 (2); Arg 3.05 (3). Mass spectrum $(M+H)^+$, found 3456.7, theoretical 3457.0

Example 12: Preparation of Val^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, trifluoroacetate salt Compound No. 12.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 2.05 (2); Thr 0.99

(1); Ser 2.46 (3); Glu 2.06 (2); Ala 3.09 (3); Val 2.02 (2); Ile 1.86 (2); Leu 5.01 (5); Tyr 1.96 (2); Phe 0.96 (1); Lys 2.00 (2); Arg 2.01 (2). Mass spectrum $(M+H)^+$, found 3213.5, theoretical 3213.8

Example 13: Preparation of Val^2 Ser^8 Ala^{15} Hse^{26} bGRF(1-26)NH_2, trifluoroacetate salt Compound No. 13.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 2.06 (2); Thr 0.92 (1); Ser 2.68 (3); Glu 2.10 (2); Ala 3.46 (3); Val 1.89 (2); Ile 0.86 (1); Leu 4.11 (4); Tyr 1.78 (2); Phe 0.82 (1); Lys 1.88 (2); Arg 1.94 (2). Mass spectrum $(M+H)^+$, found 2986.7, theoretical 2987.5

Example 14: Preparation of Val^2 Ser^8 Ala^{15} Hse^{21} bGRF(1-21)NH_2, trifluoroacetate salt Compound No. 14.

The synthesis of the GRF analog peptide having the formula: H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Hse-NH_2 (as the CF_3COOH salt) is conducted in a stepwise manner as in procedure A. Amino acid analysis, theoretical values in parentheses: Asp 1.03 (1); Thr 0.94 (1); Ser 2.77 (3); Glu 1.06 (1); Ala 3.38 (3); Val 1.86 (2); Ile 0.84 (1); Leu 2.04 (2); Tyr 1.84 (2); Phe 0.86 (1); Lys 0.93 (1); Arg 2.02 (2). Mass spectrum $(M+H)^+$, found 2389.3, theoretical 2389.0

Following the stepwise manner as in procedure A, the following peptides can also be prepared:

Ile^2 Ser^8 Ile^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2

Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2

Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_4H_9

Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5

Ile^2 Ser^8 Ala^{15} Hse^{27} bGRF(1-27)NH-C_6H_{13}

Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{29} bGRF(1-29)NH-C_8H_{17}

Thr^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2

Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5

Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2

Thr^2 Val^{15} bGRF(1-29)NH_2

Thr^2 Leu^{15} Leu^{27} bGRF(1-29)NH_2

Thr^2 Ile^{15} Leu^{27} bGRF(1-29)NH_2

Thr^2 Ser8 Val^{15} Leu^{27} Ser^{28} Hse30 bGRF(1-30)NH_2

Thr^2 Ser^8 Ala^{15} Ile^{27} Ser^{28} bGRF(1-29)NH_2
Thr^2 Ser^8 Leu^{15} Leu^{27} Ser^{28} Hse30 bGRF(1-30)OH
Thr^2 Ser^8 Ile^{15} Leu^{27} Ser^{28} bGRF(1-40)OH
Val^2 Ser^8 Val^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2
Val^2 Ser^8 Ala^{15} Ile^{27} Ser^{28} Hse37 bGRF(1-37)NH_2
Val^2 Ser^8 Leu^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2
N-α-(Tyr-Thr)$_2$-Tyr^1 Thr^2 Val^{15} Leu^{27} bGRF(1-29)NH_2
N-α-(Tyr-Ser)-Tyr^1 Thr^2 Ala^{15} Leu^{27} Hse^{30} bGRF(1-30)NH_2
N-α-(Tyr-Thr)$_2$-Tyr^1 Thr^2 Ile^{15} Leu^{27} bGRF(1-29)NH_2
N-α-(Tyr-Thr)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2
N-α-(Tyr-Ser-Tyr-Thr)-Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2
N-α-(Tyr-Thr-Tyr-Ser)-Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2
N-α-(Asp-Ala)-Tyr^1 Thr^2 Ala^{15} Leu^{27} Hse^{33} bGRF(1-33)NH-C2H_5
N-α-(Asp-Ala)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_4H_9
N-α-(Asp-Ala-Tyr-Thr)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2
Thr^2 Cya^3 Ala^{15} Leu^{27} bGRF(1-29)NH_2
Thr^2 Cya^3 Ala^{15} Ser^8 Leu^{27} Ser^{28} bGRF(1-29)NH_2
Val^2 Cya^3 Ala^{15} Leu^{27} bGRF(1-29)NH_2
Ile^2 Cya^3 Ala^{15} Leu^{27} bGRF(1-29)NH_2

PROCEDURES

PROCEDURE B

In addition to preparation of GRF analogs by solid phase methods, the analogs can be obtained by recombinant DNA methodology by the procedure described for Leu^{27} bGRF(1-44)OH (European Patent Application 0212531) with the following modifications in the segment of DNA coding for bGRF(1-44)OH the codons for Ala^2, Asn^8, Gly^{15} and Asn^{28} are replaced by the codons: for example (ACT) Thr^2 or (ATT) Ile^2 or (GTT) Val^2, (AGT) Ser^8, (GCT) Ala^{15}, (AGT) Ser^{28}, respectively.

Additionally, for Cya^3 containing GRF analogs, the codon GAT (Asp^3) is replaced by the codon TGT for Cys^3. After expression of the protein and cleavage with cyanogen bromide in formic acid as described in the above European Patent Application, hydrogen peroxide

is added to the solution at about 0°C to effect oxidation of the Cys residues to Cya. The peptide is then purified by the methods described.

Additionally, for N-terminally extended GRF analogs, the DNA segments coding for the extension are added to the N-terminus as follows: (TATACT) for Tyr-Thr, $(TATACT)_n$ for $(Tyr\text{-}Thr)_n$ or (TATAGT) for Tyr-Ser, $(TATAGT)_n$ for $(Tyr\text{-}Ser)_n$, or (TATAGTTATACT) for Tyr-Ser-Tyr-Thr or (TATACTTATAGT) for Tyr-Thr-Tyr-Ser, (GATGCT) for Asp-Ala etc. The gene for the precursor protein is inserted into an E. coli expression vector. After expression of the protein isolation of the inclusion bodies and then cleaving them with cyanogen bromide in formic acid as described in the above European Patent Application, the formic acid is removed under reduced pressure. The crude peptide is then purified by the methods described.

The ability to produce the compounds of the subject invention by known recombinant DNA technology is possible since the claimed peptides are constituted entirely of naturally occurring amino acids. This is in contrast to known analogs which contain non-DNA-coded components, such as D-Ala and/or desaminoTyr, and will require for any large scale production a costly chemical synthesis or a combination of genetic engineering with chemical procedures.

Recombinant host microorganisms used in this invention are made by recombinant DNA techniques well known to those skilled in the art and set forth, for example, in Molecular Cloning, T. Maniatis, et al., Cold Spring Harbor Laboratory, (1982) and B. Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons (1984), which are incorporated herein by reference.

C-terminal Hse(lactone), HseOH and $HseN(R_a)(R_b)$ analogs can be prepared by the methods disclosed in Kempe et al, BIO/TECHNOLOGY, Vol 4, pp 565-568 (1986).

PROCEDURE C

This procedure deals with the preparation of N-alkylated GRF analogs described in the subject invention. The peptides will be made using either chemical or biotechnology procedures (Procedure A or Procedure B, respectively). N-alkylation will then be achieved by known methods e.g. Murphy, W.A. and Coy, D.H., Potent long-acting alkylated analogs of growth hormone-releasing factor, Peptide Research 1, 36-41 (1988), V. Sythyamoorthy et al. Reductive

methylation of botulinum neurotoxin types A and B. Mol. Cell. Biochem. 83, 65-72 (1988).

Following Procedure C, the following peptides can also be prepared:

N-ε-iPr-Tyr^{1} Thr^{2} Ser^{8} N-ε-iPr-$Lys^{12,21}$ Val^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2

N-ε-iPr-Tyr^{1} Thr^{2} Ser^{8} N-ε-iPr-$Lys^{12,21}$ Ala^{15} Leu^{27} bGRF(1-40)NH_2

N-ε-Bzl-Tyr^{1} Thr^{2} Ser^{8} N-ε-Bzl-$Lys^{12,21}$ Val^{15} Ile^{27} Ser^{28} bGRF(1-32)NH_2

N-ε-iPr-Tyr^{1} Ile^{2} Ser^{8} N-ε-iPr-$Lys^{12,21}$ Ala^{15} Leu^{27} bGRF(1-40)NH_2

N-ε-Bzl-Tyr^{1} Val^{2} Ser^{8} N-ε-Bzl-$Lys^{12,21}$ Val^{15} Ile^{27} Ser^{28} bGRF(1-32)NH_2

All of the synthetic GRF peptides of the subject invention, including the peptides prepared in the Examples, are considered to be biologically active and useful for stimulating the release of GH by the pituitary.

Dosages between about 10 nanograms and about 5 micrograms of these peptides per kilogram (kg) of body weight are considered to be particulary effective in causing GH secretion.

Stimulation of GH secretion by such peptides should result in an attendant increase in growth for humans, bovine and other animals with normal GH levels. Moreover, administration should alter body fat content and modify other GH-dependent metabolic, immunologic and developmental processes. For example, these analogs may be useful as a means of stimulating anabolic processes in human beings under circumstances such as following the incurring of burns. As another example, these analogs may be administered to commercial warm-blooded animals such as chickens, turkeys, pigs, goats, cattle and sheep, and may be used in agriculture for raising fish and other cold-blooded marine animals, e.g., sea turtles and eels, and amphibians, to accelerate growth and increase the ratio of protein to fat gained by feeding effective amounts of the peptides. These analogs may be used for stimulation of the immune functions in human and animal.

Daily dosages of between 10 nanograms/Kg and about 50 micrograms/Kg body weight are considered to be particularly effective in

increasing lactation, growth and stimulating the immune functions.

For administration to humans and animals, these synthetic peptides should have a purity of at least about 93% and preferably at least 98%.

These synthetic peptides or the nontoxic salts thereof, combined with a pharmaceutically or veterinarily acceptable carrier to form a pharmaceutical composition, preferably as sustained release formulations, may be administered to animals, including humans, either intravenously, subcutaneously, intramuscularly, percutaneously, e.g. intranasally. The administration may be employed by a physician to stimulate the release of GH where the host being treated requires such therapeutic treatment. The required dosage will vary with the particular condition being treated, with the severity of the condition and with the duration of desired treatment.

Such peptides are often administered in the form of nontoxic salts, such as acid addition salts or metal complexes, e.g., with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to administered by intravenous administration in isotonic saline, phosphate buffer solutions or the like may be effected.

The peptides should be administered to humans under the guidance of a physician, and pharmaceutical compositions will usually contain the peptide in conjunction with a conventional, solid or liquid, pharmaceutically-acceptable carrier. Usually, the parental dosage will be from about 100 nanograms to about 50 micrograms of the peptide per kilogram of the body weight of the host.

Although the invention has been described with regard to its preferred embodiments, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto. For example, modifications in the peptide chain, particularly deletions of one or two residues beginning at the C-terminus of the peptide, can be made in accordance with known experimental practices to date to create peptides that retain very substantial portions of the

biological potency of the peptide, and such peptides are considered as being within the scope of the invention. Moreover, additions may be made to the C-terminus, and/or to the N-terminus, and/or generally equivalent residues can be substituted for naturally occurring residues, as is known in the overall art of peptide chemistry, to produce other analogs, having increased resistance to proteolysis, for example, and also having at least a substantial portion of the potency of the claimed polypeptide, without deviating from the scope of the invention, such as those illustrated by Compounds 1-14. Likewise known substitutions in the carboxyl moiety at the C-terminus, e.g. a lower alkyl amide, also produce equivalent molecules.

In the same manner as disclosed herein,

GRF PEPTIDES of the formula $R'-R_1-R'_2-R_3$-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-$R'_{12}-R_{13}$-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-$R'_{21}-R_{22}$-Leu-Gln-R_{25}-Ile-$R_{27}-R_{28}$-Arg-Gln-Gln-Gly-Glu-$R_{34}-R_{35}$-Gln-Glu-$R_{38}-R_{39}-R_{40}$-Arg-R_{42}-Arg-Leu-Z

wherein

R' is $H-(Y-X)_n$ or $H-(Y-X)_m(Y'-X')_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr or Ser, or when Y or Y' is Asp, then X and X' is Ala; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His;

R'_2 is Ala, D-Ala, Thr, Val or Ile;

R_3 is Asp or Cya;

R_8 is Asn or Ser;

R'_{12} is Lys or Arg;

R_{13} is Val or Ile;

R_{15} is Ala, Val, Leu, Ile or Gly;

R_{18} is Ser or Tyr;

R'_{21} is Lys or Arg;

R_{22} is Ala or Leu;

R_{25} is Asp, Glu or Cya;

R_{27} is Met, Ile or Leu;

R_{28} is Asn or Ser;

R_{34} is Ser or Arg;

R_{35} is Asn or Ser;

R_{38} is Arg or Gln;

R_{39} is Gly or Arg;

R_{40} is Ala or Ser;

R_{42} is Ala, Val or Phe; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical $-COOR_a$, $-CR_aO$, $-CONHNHR_a$, $-CON(R_a)(R_b)$ or $-CH_2OR_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 (preferably positions 27 through 44) as its C-terminus; or a Hse(lactone), HseOH or $HseN(R_a)(R_b)$ of the foregoing and/or a non-toxic salt of the foregoing; can be made in accordance with known experimental practices to date to create peptides that retain very substantial portions of the biological potency of the peptide, and such peptides are considered as also being another aspect of the invention disclosed herein. These peptides can be prepared (substituting N-Methyl-D-Ala, D-Ala or preferably Ala at the 2 position for Val, Ile or Thr) and can be utilized in the same general manner.

TABLE I

In vitro stability in bovine plasma and in vivo bioactivity in steer of GRF analogs substituted at position 2 and 15.

Compound+	In vitro stability† (% Inact peptide after 1 hr)	In vivo relative potency iv#	sc§
	STUDY I		
bGRF(1-44)NH2	nd	100^a	
Leu27-bGRF(1-29)NH2	39.8*	106^a	
Ala15,Leu27-bGRF(1-29)NH2	56.2*	118^a	
Thr2,Leu27-bGRF(1-29)NH2++	75.6*	94^a	
Thr2,Ala15,Leu27-bGRF(1-29)NH2++	91.1*	195^b	
	STUDY II		
bGRF(1-44)NH2	nd	100^a	100^a
Leu27-bGRF(1-29)NH2	46.8**	nd	nd
Leu2,Ala15,Leu27-bGRF(1-29)NH2++	86.3**	100^a	nd
Val2,Ala15,Leu27-bGRF(1-29)NH2++	83.4**	167^b	214^b
Ile2,Ala15,Leu27-bGRF(1-29)NH2++	80.3**	171^b	162^c
Thr2,Ala15,Leu27-bGRF(1-29)NH2++	nd	148^b	187^b

† Peptides at 0.03 mM were incubated with bovine plasma at 37°C for one hr. Aliquots of 0.1 ml were quenched with 2 ml of 0.2% aqueous trifluoroacetic acid solution and subjected to solid phase extraction and HPLC analysis as described in T.M. Kubiak et al. Drug Met. Disp. 17, 393-397 (1989).

Steers were injected iv with peptides at the dose of 0.01 nmol/kg and the procedures were as described in W.M. Moseley et al. J. Endocr. 117, 253-259 (1988). Relative potency was calculated on the basis of the area under serum growth hormone (GH) curve monitored for 2 hrs after treatment.

§ Steers were injected subcutaneously with peptides at the dose of 0.75 nmol/kg. Relative potency was calculated on the basis of the area under serum GH curve monitored for 6 hrs after treatment.
a,b,c values with different superscripts within each column and/or study were statistically different at $p < 0.05$.

nd = not determined

* Plasma pool 1

**Plasma pool 2, different from 1

\+ as the trifluoroacetate salt

++ No cleavage after residue 2 was observed upon incubation in bovine plasma in vitro over a 1 hr incubation period.

CLAIMS

1. A GRF PEPTIDE having Thr, Val or Ile residue in place of the amino acid residue normally found at position 2 and having an amino acid selected from the group consisting of Ala, Val, Leu, Ile or Gly at position 15, optionally substituted at the N-terminus with H-(Y-X)$_n$ or H-(Y-X)$_m$(Y'-X')$_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr, Ser or Ala; n is 1-10; m is 1-5; and p is 1-5.

2. A GRF PEPTIDE of Claim 1 wherein the amino acid at position 15 is Ala.

3. A GRF PEPTIDE of Claim 1 having the formula R-R_1-R_2-R_3-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-R_{12}-R_{13}-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-R_{21}-R_{22}-Leu-Gln-R_{25}-Ile-R_{27}-R_{28}-Arg-Gln-Gln-Gly-Glu-R_{34}-R_{35}-Gln-Glu-R_{38}-R_{39}-R_{40}-Arg-R_{42}-Arg-Leu-Z

wherein

R is H, C_1-C_5 alkyl, benzyl, H-(Y-X)$_n$ or H-(Y-X)$_m$(Y'-X')$_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr, Ser or Ala; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His;

R_2 is Thr, Val or Ile;

R_3 is Asp or Cya;

R_8 is Asn or Ser;

R_{12} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{13} is Val or Ile;

R_{15} is Ala, Val, Leu, Ile or Gly;

R_{18} is Ser or Tyr;

R_{21} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{22} is Ala or Leu;

R_{25} is Asp or Glu;

R_{27} is Met, Ile or Leu;

R_{28} is Asn or Ser;

R_{34} is Ser or Arg;

R_{35} is Asn or Ser;

R_{38} is Arg or Gln;

R_{39} is Gly or Arg;

R_{40} is Ala or Ser;

R_{42} is Ala, Val or Phe; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical $-COOR_a$, $-CR_aO$, $-CONHNHR_a$, $-CON(R_a)(R_b)$ or $-CH_2OR_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 as its C-terminus; or a Hse(lactone), HseOH or $HseN(R_a)(R_b)$ of the foregoing and/or a non-toxic salt of the foregoing.

4. A GRF PEPTIDE of Claim 3 having the formula H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Hse-NH_2 or a non-toxic salt thereof.

5. A GRF PEPTIDE of Claim 3 having the formula H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Hse-NH_2 or a non-toxic salt thereof.

6. A GRF PEPTIDE of Claim 3 comprising a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 27 through 44 as its C-terminus, and wherein R_{15} is Ala, Val, Leu or Ile.

7. A GRF PEPTIDE of Claim 6 wherein R is H and R_{15} is Ala.

8. A GRF PEPTIDE of Claim 7 wherein R_1 is Tyr.

9. A GRF PEPTIDE of Claim 8 wherein R_8 and R_{28} are Ser.

10. A GRF PEPTIDE of Claim 9 having the formula H-Tyr-Val-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Hse-NH_2 or a non-toxic salt thereof.

11. A GRF PEPTIDE of Claim 6 wherein R_2 is Thr.

12. A GRF PEPTIDE of Claim 11 having the formula H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Hse-NH_2, H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Gln-Gln-Gly-Hse-NH-C_2H_5, H-Tyr-Thr-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Hse-NH_2, or a non-toxic salt thereof.

13. A GRF PEPTIDE of Claim 6 wherein R_2 is Ile.

14. A GRF PEPTIDE of Claim 13 having the formula H-Tyr-Ile-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-Hse-NH_2 or a non-toxic salt thereof.

15. A GRF PEPTIDE of Claim 6 selected from the group consisting of Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1-30)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{33} bGRF(1-33)NH-C_2H_5, Val^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, Ile^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Ile^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, Thr^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH_2, Thr^2 Ser^8 Ala^{15} Leu^{27} Hse^{28} bGRF(1-28)NH-C_2H_5, or a non-toxic salt thereof.

16. A GRF PEPTIDE of Claim 6 selected from the group consisting of Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, Val^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, Ile^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Thr)Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-$(Tyr\text{-}Thr)_2Tyr^1$ Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Thr)Tyr^1 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Thr)Tyr^1 Ile^2 Ala^{15}

Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Ser)Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, Val^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{45} bGRF(1-45)NH2, Ile^2 Ser^8 Ala^{15} Hse^{27} bGRF(1-27)NH_2, Thr^2 Val^{15} bGRF(1-29)NH_2, Thr^2 Leu^{15} Leu^{27} bGRF(1-29)NH_2, Thr^2 Ile^{15} Leu^{27} bGRF(1-29)NH_2, Thr^2 Ser8 Val^{15} Leu^{27} Ser^{28} Hse30 bGRF(1-30)NH_2, Thr^2 Ser^8 Ala^{15} Ile^{27} Ser^{28} bGRF(1-29)NH_2, Thr^2 Ser^8 Leu^{15} Leu^{27} Ser^{28} Hse30 bGRF(1-30)OH, Thr^2 Ser^8 Ile^{15} Leu^{27} Ser^{28} bGRF(1-40)OH, N-α-$(Tyr-Thr)_2$-Tyr^1 Thr^2 Val^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Ser)-Tyr^1 Thr^2 Ala^{15} Leu^{27} Hse^{30} bGRF(1-30)NH_2, N-α-$(Tyr-Thr)_2$-Tyr^1 Thr^2 Ile^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Thr)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2, N-α-(Tyr-Ser-Tyr-Thr)-Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Tyr-Thr-Tyr-Ser)-Tyr^1 Thr^2 Ala^{15} Leu^{27} bGRF(1-29)NH_2, N-α-(Asp-Ala)-Tyr^1 Thr^2 Ala^{15} Leu^{27} Hse^{33} bGRF(1-33)NH-C2H_5, N-α-(Asp-Ala)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} Hse^{30} bGRF(1- 30)NH-C_4H_9, N-α-(Asp-Ala-Tyr-Thr)-Tyr^1 Thr^2 Ser^8 Ala^{15} Leu^{27} Ser^{28} bGRF(1-29)NH_2, or a non-toxic salt thereof.

17. A method of stimulating the release of growth hormone in an animal, which comprises administering to said animal an effective amount of a GRF PEPTIDE having Thr, Val or Ile residue in place of the amino acid residue normally found at position 2 and having an amino acid selected from the group consisting of Ala, Val, Leu, Ile or Gly at position 15, optionally substituted at the N-terminus with $H\text{-}(Y\text{-}X)_n$ or $H\text{-}(Y\text{-}X)_m(Y'\text{-}X')_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr, Ser or Ala; n is 1-10; m is 1-5; and p is 1-5.

18. A method according to Claim 17 wherein the GRF PEPTIDE has the formula R-R_1-R_2-R_3-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-R_{12}-R_{13}-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-R_{21}-R_{22}-Leu-Gln-R_{25}-Ile-R_{27}-R_{28}-Arg-Gln-Gln-Gly-Glu-R_{34}-R_{35}-Gln-Glu-R_{38}-R_{39}-R_{40}-Arg-R_{42}-Arg-Leu-Z

wherein

R is H, C_1-C_5 alkyl, benzyl, $H\text{-}(Y\text{-}X)_n$ or $H\text{-}(Y\text{-}X)_m(Y'\text{-}X')_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr, Ser or Ala; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His;

R_2 is Thr, Val or Ile;

R_3 is Asp or Cya;

R_8 is Asn or Ser;

R_{12} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{13} is Val or Ile;

R_{15} is Ala, Val, Leu, Ile or Gly;

R_{18} is Ser or Tyr;

R_{21} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{22} is Ala or Leu;

R_{25} is Asp or Glu;

R_{27} is Met, Ile or Leu;

R_{28} is Asn or Ser;

R_{34} is Ser or Arg;

R_{35} is Asn or Ser;

R_{38} is Arg or Gln;

R_{39} is Gly or Arg;

R_{40} is Ala or Ser;

R_{42} is Ala, Val or Phe; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical $-COOR_a$, $-CR_aO$, $-CONHNHR_a$, $-CON(R_a)(R_b)$ or $-CH_2OR_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 as its C-terminus; or a Hse(lactone), HseOH or $HseN(R_a)(R_b)$ of the foregoing and/or a non-toxic salt of the foregoing.

19. A method according to Claim 17 wherein the GRF PEPTIDE consists of a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 27 through 44 as its C-terminus.

20. A composition for stimulating the release of growth hormone in an animal comprising an effective amount of a GRF PEPTIDE of the formula R-R_1-R_2-R_3-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-R_{12}-R_{13}-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-R_{21}-R_{22}-Leu-Gln-R_{25}-Ile-R_{27}-R_{28}-Arg-Gln-Gln-Gly-Glu-R_{34}-R_{35}-Gln-Glu-R_{38}-R_{39}-R_{40}-Arg-R_{42}-Arg-Leu-Z

wherein

R is H, C_1-C_5 alkyl, benzyl, H-(Y-X)$_n$ or H-(Y-X)$_m$(Y'-X')$_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr, Ser or Ala; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His;

R_2 is Thr, Val or Ile;

R_3 is Asp or Cya;

R_8 is Asn or Ser;

R_{12} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{13} is Val or Ile;

R15 is Ala, Val, Leu or Ile;

R_{18} is Ser or Tyr;

R_{21} is Lys or Arg, or N-ε-alkyl- or N-ε-benzyl-Lys when R is C_1-C_5 alkyl or benzyl;

R_{22} is Ala or Leu;

R_{25} is Asp or Glu;

R_{27} is Met, Ile or Leu;

R_{28} is Asn or Ser;

R_{34} is Ser or Arg;

R_{35} is Asn or Ser;

R_{38} is Arg or Gln;

R_{39} is Gly or Arg;

R_{40} is Ala or Ser;

R_{42} is Ala, Val or Phe; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical -COOR$_a$, -CR$_a$O, -CONHNHR$_a$, -CON(R$_a$)(R$_b$) or -CH$_2$OR$_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 as its C-terminus; or a Hse(lactone), HseOH or HseN(R$_a$)(R$_b$) of the foregoing and/or a non-toxic salt of the foregoing; in association with a pharmaceutically acceptable carrier.

21. A composition according to Claim 19 wherein the GRF PEPTIDE consists of a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 27 through 44 as its C-terminus.

22. A GRF PEPTIDE of the formula R'-R_1-R'$_2$-R_3-Ala-Ile-Phe-Thr-R_8-Ser-Tyr-Arg-R'$_{12}$-R_{13}-Leu-R_{15}-Gln-Leu-R_{18}-Ala-Arg-R'$_{21}$-R_{22}-Leu-Gln-R_{25}-Ile-R_{27}-R_{28}-Arg-Gln-Gln-Gly-Glu-R_{34}-R_{35}-Gln-Glu-R_{38}-R_{39}-R_{40}-Arg-R_{42}-Arg-Leu-Z

wherein

R' is H-$(Y\text{-}X)_n$ or H-$(Y\text{-}X)_m(Y'\text{-}X')_p$ wherein Y and Y', being the same or different, is a naturally occurring amino acid; X and X', being the same or different, is selected from Thr or Ser, or when Y or Y' is Asp, then X and X' is Ala; n is 1-10; m is 1-5; p is 1-5;

R_1 is Tyr or His;

R'$_2$ is Ala, D-Ala, Thr, Val or Ile;

R_3 is Asp or Cya;

R_8 is Asn or Ser;

R'$_{12}$ is Lys or Arg;

R_{13} is Val or Ile;

R_{15} is Ala, Val, Leu, Ile or Gly;

R_{18} is Ser or Tyr;

R'$_{21}$ is Lys or Arg;

R_{22} is Ala or Leu;

R_{25} is Asp, Glu or Cya;

R_{27} is Met, Ile or Leu;

R_{28} is Asn or Ser;

R_{34} is Ser or Arg;

R_{35} is Asn or Ser;

R_{38} is Arg or Gln;

R_{39} is Gly or Arg;

R_{40} is Ala or Ser;

R_{42} is Ala, Val or Phe; and

Z signifies the carboxyl moiety of the amino acid residue at the C-terminal and is the radical $-COOR_a$, $-CR_aO$, $-CONHNHR_a$, $-CON(R_a)(R_b)$ or $-CH_2OR_a$, with R_a and R_b being C_1-C_8 alkyl or hydrogen; or a biologically active fragment thereof extending from R at the N-terminus to a residue in any of positions 20 through 44 as its C-terminus; or a Hse(lactone), HseOH or HseN$(R_a)(R_b)$ of the foregoing and/or a non-toxic salt of the foregoing.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02923

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/10 ; A61K37/43

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	EP,A,320785 (HOECHST AKTIENGESELLSCHAFT) 21 June 1989 see claim 1 ---	1
A	EP,A,136475 (ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 10 April 1985 see claim 1 ---	1, 3
A	EP,A,307860 (F. HOFFMANN-LA ROCHE AG) 22 March 1989 see claim 13 ---	1-2

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 AUGUST 1990	12. 09. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BEVAN S.R.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/02923

SA 37422

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/08/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-320785	21-06-89	DE-A- 3742633	29-06-89
		AU-A- 2684388	22-06-89
		JP-A- 1199997	11-08-89
EP-A-136475	10-04-85	AU-B- 575843	11-08-88
		AU-A- 3133484	14-02-85
		CA-A- 1268898	08-05-90
		JP-A- 60057000	02-04-85
EP-A-307860	22-03-89	AU-A- 2234688	23-03-89
		JP-A- 1129000	22-05-89

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82